Supplemental Information Disclosure Statement References C4 and C11

Applicant encloses herewith additional, legible copies of references C4 and C11 submitted in the Supplemental Information Disclosure Statement of December 9, 2005. Applicant respectfully requests consideration of these references by the Examiner.

Rejection of Claims 114-121 and 141-166 Under Nonstatutory Obviousness-Type Double Patenting

Claims 141-142 and 159-166 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 36-40 and 49 of U.S. Patent No. 6,509,015. In addition, claims 114-121, 143-150, and 151-158 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 4-7 and 36-39 of U.S. Patent No. 6,509,015.

While in no way admitting that the present claims are obvious over claims 4-7, 36-40 and 49 of U.S. Patent No. 6,509,015, Applicant respectfully submits that upon indication of allowable subject matter, Applicant will file a terminal disclaimer in compliance with 37 C.F.R. 1.321(b) and (c), if appropriate, and will obviate the rejection of the pending claims in view of the cited claims of U.S. Patent No. 6,509,015.

SUMMARY

In view of the foregoing remarks, reconsideration of the rejections and allowance of all pending claims is respectfully requested. If a telephone conversation with Applicant's Attorney would expedite the prosecution of the above-identified application, the Examiner is urged to call Applicant's Attorney at (617) 227-7400.

Respectfully submitted,

Elizabeth A. Hanley, Esq.
Registration No. 33,505
Attorney for Applicants

LAHIVE & COCKFIELD, LLP
28 STATE STREET
BOSTON, MA 02109
(617) 227-7400 (Telephone)

Dated: **August 9, 2006**

C4

LIPPINCOTT WILLIAMS & WILKINS

Critical Care Medicine
OFFICIAL JOURNAL OF THE SOCIETY OF CRITICAL CARE MEDICINE

Critical Care Medicine: Volume 24(9) September 1996 pp 1431-1440

INTERSEPT: An international, multicenter, placebo-controlled trial of monoclonal antibody to human tumor necrosis factor-alpha in patients with sepsis [Feature Article]

Cohen, Jonathan MB FRCP; Carlet, Jean MD

for the INTERSEPT Study Group*; From the Department of Infectious Diseases and Bacteriology, Hammersmith Hospital and Royal Postgraduate Medical School, London, UK (Dr. Cohen); and the Department of Critical Care Medicine, Hopital St. Joseph, Paris, France (Dr. Carlet).

*Participating investigators and collaborators are listed in *Table 9*.

Supported, in part, by Bayer Corporation. Drs. G. Arcieri, G. Lemm, and B. Sommerauer are employees of Bayer Corporation.

Address requests for reprints to: Prof. J. Cohen, Department of Infectious Diseases and Bacteriology, Hammersmith Hospital and Royal Postgraduate Medical School, Du Cane Road, London W12 0NN, UK.

Abstract TOP

Objective: To determine the safety and efficacy of BAY times 1351, a murine monoclonal antibody to recombinant human tumor necrosis factor (TNF)-alpha, in patients with sepsis.

Design: An international, multicenter, prospective, placebo-controlled trial in patients with sepsis, stratified into shock/nonshock groups.

Setting: Forty acute clinical care facilities in 14 countries.

Patients: Of the 564 patients enrolled in the study, 553 patients received study drug or placebo.

Interventions: Patients received 15 mg/kg or 3 mg/kg of BAY times 1351, or placebo, as a single intravenous infusion.

Measurements and Main Results: The patients were well matched for severity of illness and for risk factors known to influence the outcome of sepsis. There was no difference in 28-day mortality rates between groups (placebo group 66 [39.5%] of 167; 3 mg/kg group 57 [31.5%] of 181; 15 mg/kg group 87 [42.4%] of 205). Approximately 9 months after this study had begun, an interim safety examination of NORASEPT, a North American Sepsis Trial using the same monoclonal antibody, indicated that there was no benefit to patients in the nonshock group and further enrollment of these nonshock septic patients into INTERSEPT was stopped. The analysis therefore focused on the 420 patients

Article Outline

in shock. The primary efficacy variable was the 28-day, all-cause mortality rate: placebo group 57 (42.9%) of 133; 3-mg/kg group 51 (36.7%) of 139; and 15-mg/kg group 66 (44.6%) of 148 (not significant). Two secondary efficacy variables were identified prospectively: shock reversal and frequency of organ failures. Life-table analysis showed that in patients who survived 28 days, there was a more rapid reversal of shock in both treatment groups compared with placebo (15-mg/kg group vs. placebo group log-rank statistic p = .007, 3-mg/kg group vs. placebo group p = .01). Similarly, in patients surviving 28 days, there was a significant delay in the time to the onset of first organ failure (log rank 15 mg/kg vs. placebo p = .03, 3 mg/kg vs. placebo p = .07), and more patients in the placebo group developed at least one organ failure: 15-mg/kg group 33 (40.2%) of 82; 3-mg/kg group 39 (44.3%) of 88; and placebo group 45 (59.2%) of 76 (15 mg/kg vs. placebo p = .03, 3 mg/kg vs. placebo p = .06). No significant adverse events were associated with the monoclonal antibody treatment.

Conclusions: INTERSEPT provides additional clinical data implicating TNF-alpha as an integral mediator of septic shock. The study suggested a possible role for anti-TNF antibody as adjunctive therapy, but this possibility requires confirmation by another clinical trial.

- Table 1
- Figure 1
- Table 2
- Table 3
- Table 4
- Figure 2
- Table 5
- Table 6
- Figure 3
- Table 7
- Table 8
- Table 9

(Crit Care Med 1996; 24:1431-1440)

Sepsis is a syndrome characterized by infection, organ hypoperfusion, and a mortality rate of approximate 35% [1]. Despite the fact that the process is initiated by infection, conventional antimicrobial therapy has had only limited success in reducing this mortality rate, in part because much of the tissue injury results from inappropriate and uncontrolled host responses. During the last 10 yrs, it has become clear that microbial components, such as endotoxin (lipopolysaccharide), activate a complex network of mediator cascades, and it is these mediators that, directly or indirectly, cause organ failure [2]. The recognition of the role of these systems in causing the sepsis syndrome has been the impetus behind attempts to develop novel approaches to therapy based on neutralizing or subverting these inappropriate responses [3].

Several lines of evidence suggested that tumor necrosis factor (TNF)-alpha was a particularly important mediator of sepsis. TNF-alpha has been found in the serum of septic patients, and has been, at least in some cases, correlated with the severity of this disease [4,5]. Injection of endotoxin in humans caused an increase in circulating TNF-alpha concentrations coincident with physiologic changes such as fever and tachycardia [6], and, in animals, injection of TNF-alpha reproduced the hemodynamic features seen in septic patients [7]. Finally, in a variety of experimental models, antibodies to TNF-alpha prevented death after endotoxin or live bacterial challenge [8-12]. These and other data set the scene for studies of anti-TNF antibodies in patients.

Several phase I and II studies [13-16] showed that murine monoclonal antibodies to TNF-alpha could be given safely to patients with sepsis, and these studies provided pharmacokinetic data that were helpful in determining an appropriate dose. The results were sufficiently encouraging that two phase II/III trials were initiated. Both studies used the same antibody, BAY times 1351, a murine monoclonal antibody to TNF-alpha, and both used the same study design in which two treatment groups were compared with a placebo. However, in the first study [17], called NORASEPT (North American Sepsis Trial), the treatment groups received either 7.5 or 15 mg/kg of the antibody, while in INTERSEPT (International Sepsis Trial), the 7.5-mg/kg dose was replaced by a 3-mg/kg dose. In all other respects, the protocols used for the two studies were the same.

MATERIALS AND METHODS TOP

Study Design. TOP

The study was an international, multicenter, prospective, randomized, placebo-controlled double-blind trial of anti-TNF antibody in patients with sepsis. It started in May 1991, 6 months after the start of NORASEPT, and finished in July 1993. The protocol was approved by the Institutional Review Boards of each participating center, and by the independent European Ethical Review Committee, Leuven, Belgium. Informed consent was obtained from all patients or their relatives. During the entire period of evaluation, patients received full intensive care management, including fluid resuscitation, vasopressors, ventilatory support, parenteral antimicrobial agents, and appropriate

surgical management, including drainage of septic foci. Patients who satisfied the entry criteria were stratified according to whether they had shock. Shock was defined as a sustained decrease in systolic blood pressure to <90 mm Hg, or a decrease of 40 mm Hg from the baseline for at least 30 mins and no response to a fluid challenge, with the provision that there was an absence of other causes of shock, such as hypovolemia or myocardial infarction. Shock was considered present as long as vasopressor therapy was required to maintain the systolic pressure at >or=to90 mm Hg. A random number generator was used to produce a stratified randomization in blocks of 12 at each center. Because randomization was in blocks, centers enrolling <11 patients by chance enrolled more high-dose patients. When a patient was identified as suitable for enrollment, the study drug was prepared by the pharmacist, who was the only individual who was aware of the treatment allocation. Patients were assigned to receive a single 100-mL dose of one of the three treatment regimens (high-dose 15 mg/kg, low-dose 3 mg/kg, or placebo) by intravenous infusion over 30 mins. Patients were assessed until death or for the following 28 days, whichever was sooner.

Approximately 9 months after the study had begun, a planned interim safety analysis of the NORASEPT trial indicated that patients in the nonshock stratum showed no evidence of benefit, and that there was a slightly higher mortality rate in the high-dose group than in the placebo group, although this finding was not statistically significant. When these data were made available to INTERSEPT investigators, it was concluded that further enrollment of patients in the nonshock stratum would be inappropriate, and therefore, from July 22, 1992, we enrolled only patients in shock.

Patient Selection. TOP

The enrollment criteria were based on the work of Bone et al. [18], and required evidence of all of the following: acute infection; alterations in temperature; heart rate and respiratory rate; and inadequate organ perfusion. In an effort to study as homogeneous a group as possible, all patients were required to have had an acute change from baseline observations over a period not exceeding 12 hrs before enrollment. The specific inclusion criteria were: clinical evidence of acute infection; temperature of >38.3 degrees C or <35.6 degrees C; tachycardia of >90 beats/min; tachypnea of >20 breaths/min (or mechanical ventilation); and evidence of an acute change in organ function. These criteria included alteration in mental status (Glasgow Coma Scale score of <14 or acute change from baseline), hypoxemia when not due to pneumonia (PaO_2 <75 torr [<10 kPa] or PaO_2/FIO_2 <or=to37 torr [<or=to4.9 kPa] or an acute decrease of >15 torr [>2 kPa]), increased plasma lactate concentration (or pH <7.3 or base deficit >or=to10 mmol/L), oliguria (<30 mL/hr or <0.5 mL/kg/hr) or disseminated intravascular coagulation (decrease in platelets of >or=to25% plus >20% prolongation in prothrombin time, or prolongation in partial thromboplastin time combined with an increase in fibrin split products of >or=to20% or a concentration of >500 ng/mL D-dimers). Patients were excluded for any of the following reasons: a) <18 yrs of age; b) pregnant or nursing; c) uncontrolled hemorrhage or extensive burns (>20% of total surface area); and d) had, or were likely to develop, a granulocyte count of <0.1 times 10^9 neutrophils/L. Patients who had received pentoxifylline during the preceding 48 hrs, or who had received >0.5 mg/kg/day of prednisone (or glucocorticoid equivalent), were excluded from the study.

Study Drug. TOP

BAY times 1351 (Bayer Corporation, Berkeley, CA) is an immunoglobulin G_1 murine monoclonal antibody to TNF-alpha that was purified from murine hybridoma culture harvests by cell separation, polyethylene glycol precipitation, anion exchange chromatography, and size exclusion chromatography. The final product was >99% pure, with fully functional binding to human TNF-alpha in vitro, and neutralization of TNF-mediated effects in an endotoxemic baboon model [19]. Human albumin (0.25%) was used as the placebo. BAY times 1351 and placebo were provided as sterile, lyophilized powders containing glycine and maltose. The preparations were reconstituted with sterile water for injection, and the correct dosage was determined by an unblinded pharmacist, who was not involved in any aspect of patient management.

Clinical Evaluation. TOP

Vital signs were monitored at the following time periods: during the infusion; 8 hourly, for the first 4 days after infusion; and daily thereafter. The Acute Physiology and Chronic Health Evaluation (APACHE) II score [20] was calculated at randomization, and samples were obtained for bacteriological examination and for hematological and biochemical measurements. A blood sample was obtained before the study drug infusion for cytokine analysis.

Investigators classified the severity of the underlying disease conventionally, according to the criteria of McCabe and Jackson [21]. Organ/system failure was assessed in line with recommendations by Tran et al [22]. In addition, an independent efficacy and safety committee, composed of both critical care and infectious disease physicians,

evaluated each case record form to determine whether antimicrobial therapy had been appropriate. This determination was based on a comprehensive review of the susceptibility of the organisms(s) causing the infection (s), the type and dosage of the antimicrobial therapy given, and the medical and surgical management of the infective condition.

Measurement of Tumor Necrosis Factor-alpha and Interleukin-6. TOP

TNF-alpha concentrations were measured by enzyme-linked immunosorbent assay (Medgenix, Fleurus, Belgium), following the manufacturer's recommendations. The lower limit of sensitivity was 15 pg/mL. In vitro studies with pooled normal human plasma confirmed that the detection of spiked recombinant TNF-alpha was unaffected by the presence of recombinant, soluble, p55 TNF receptor. The addition of 200 micro gram/mL of BAY times 1351 inhibited 95% to 99% of TNF-alpha induced by 20 micro gram/mL of endotoxin from human whole blood in vitro.

Interleukin (IL)-6 concentrations were determined by bioassay [23], with a lower limit of detection of 40 to 60 pg/mL.

Analytical Plan. TOP

The primary efficacy variable was deemed to be the time from study drug infusion to death from all causes at 28 days, by log-rank analysis. Within each planned analysis, a Bonferroni-Holm procedure was used to account for the double comparison of low-dose and high-dose vs. placebo. To determine sample size, we assumed a 26% placebo mortality rate [18] and calculated that enrolling 960 patients would have 80% power at p = .023 for detecting a 40% reduction in mortality rate.

Secondary efficacy variables were defined in patients who survived to day 7, and in the subset of this group who survived to day 28. We wished to determine the proportion of patients who had no organ failure at the time of infusion and in whom organ failure subsequently developed, and also patients who had either shock or organ failure that subsequently reversed.

The statistical analysis was planned prospectively and was carried out on the intent-to-treat population (i.e., on all randomized patients who received the study drug). All statistical tests were two tailed. No adjustments were made for multiple comparisons of secondary efficacy variables. Therefore, reported p values should be interpreted with caution. Continuous variables were analyzed by analysis of variance or Kruskal-Wallis tests. Categorical data were analyzed by Cochran-Mantel-Haenszel tests. Time to an event was analyzed by survival analyses (log rank and Wilcoxon scores). Because this was a multicenter study, all tests (except nonparametric procedures) were adjusted for geographical region (a region was a cluster of centers) by adding region as an additional dimension to the contingency tables and generating a summary statistic. The population of all infused patients was also adjusted for the shock status at time of enrollment. Primary and secondary variables were also tested without adjustment for region, and the results are provided in this article as supportive analyses.

Since the TNF-alpha and IL-6 concentrations were not normally distributed, the data are presented as median (25% to 75% range).

RESULTS TOP

All Infused Patients. TOP

The decision in July 1992 to halt enrollment of patients in the nonshock system meant that fewer patients were enrolled in the study than the 960 originally planned. Thus, at the end of the study, we had enrolled 564 patients from 40 centers in 14 countries. Eleven patients did not receive the study drug because it was not available in time, consent was withdrawn, or a pregnancy test was positive. Monoclonal antibody was given as a 15-mg/kg dose to 205 (37.1%) patients and as a 3-mg/kg dose to 181 (32.7%) patients, while 167 (30.2%) patients were given placebo. There were no significant differences between the three groups with respect to age, gender distribution, nature or severity of underlying disease, or microbiological characteristics of the infections, indicating that they were well matched in terms of those factors recognized as having an important influence on the outcome of sepsis. The baseline APACHE II scores were 22.2 +/- 7.3 (SD), 21.4 +/- 7.7, and 23.3 +/- 7.5, respectively, for the high-dose, low-dose, and placebo groups. The difference between the low-dose and placebo groups was statistically significant at p = .03. Of particular note, we obtained preinfusion plasma from 504 (91%) patients for measurement of cytokine concentrations. We found that 86.4% of patient samples had a baseline plasma TNF-alpha concentration of >or=to15 pg/mL, and 93.7% of patients had a baseline IL-6 concentration of >or=to60 pg/mL. Both preinfusion and

1-hr postinfusion plasma TNF-alpha concentrations were available in 178, 157, and 153 patients in the 15 mg/kg, 3 mg/kg, and placebo groups, respectively. The median (25% to 75% range) preinfusion plasma TNF-alpha concentrations were 102 (39 to 281), 69 (30 to 206), and 68 (38 to 166) pg/mL, for the 15 mg/kg, 3 mg/kg, and placebo groups, respectively. One hour after infusion, the TNF-alpha concentration had decreased significantly to 7.5 (7.5 to 7.5) and 7.5 (7.5 to 54) pg/mL for the high-dose and low-dose groups, respectively, but was unchanged in the placebo group at 70 (38 to 168) pg/mL ($p = .0001$ for each comparison). The reduction in plasma TNF concentrations was significantly greater in both treatment groups compared with placebo ($p = .0001$). Furthermore, the decrease in the 15-mg/kg group was significantly ($p = .0001$) greater than that decrease seen in the 3-mg/kg group. Similarly, IL-6 concentrations were available in 185, 165, and 153 patients, and preinfusion concentrations were 2.41 (0.75 to 18.35), 2.1 (0.63 to 12.44), and 2.13 (0.62 to 13.16) ng/mL, respectively. The 1-hr postinfusion IL-6 concentrations were not affected.

The 28-day, all-cause mortality rate for the cohort of 553 infused patients is shown in Table 1. The overall placebo mortality rate was 39.5% (66/167). The mortality rate in the 3-mg/kg group was 31.5% (57/181), a reduction of 20.3%. In the 15-mg/kg group, the mortality rate was 42.4% (87/205), an increase of 7.3%. Neither of these changes was statistically significant. There were no significant differences between the survival rate curves of the three groups (log-rank analysis 3 mg/kg vs. placebo, $p = .19$) (Figure 1, top). Life-sustaining measures were either withheld or withdrawn from 14, 12, and 9 patients in the high-dose, low-dose, and placebo groups, respectively; all of these patients died.

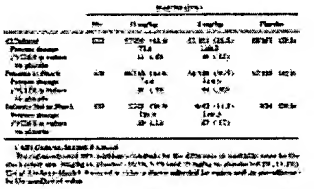


Table 1. Twenty-eight-day, all-cause mortality rates in patients who received 15 c of murine monoclonal antibody to recombinant human tumor necrosis factor-alph times 1351) or placebo.

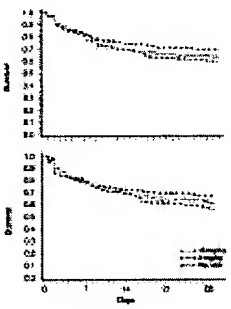


Figure 1. Top: Kaplan-Meier survival plot for all infused patients. The p values, adjusted (and unadjusted) for region, were: 15 mg/kg vs. placebo--log rank 0.48 (0.52), Wilcoxon 0.54 (0.59); 3 mg/kg vs. placebo--log rank 0.41 (0.19), Wilcoxon 0.39 (0.22). Bottom: Kaplan-Meier survival plot for patients in shock. The p values, adjusted (and unadjusted) for region (a region is a cluster of centers), were: 15 mg/kg vs. placebo--log rank 0.69 (0.77), Wilcoxon 0.69 (0.82); 3 mg/kg vs. placebo--log rank 0.63 (0.38), Wilcoxon 0.62 (0.48).

Patients in Shock: Demographics. TOP

There were 420 patients in shock at the time of randomization. Detailed comparison of factors that influence outcome from sepsis showed that the groups were well balanced Table 2. The mean duration of shock before infusion for the high-dose, low-dose, and placebo-treated patients was 11.4, 10.1, and 9.8 hrs, respectively ($p = .86$). The three most common sites of infection were the lower respiratory tract (23.5%), intra-abdominal cavity (23.4%), and bloodstream (22.8%), and the distribution of the infection sites did not differ between groups Table 3. The infection site was microbiologically documented in 76.7% of patients. For all treatment groups combined, 29.2% of patients had a Gram-negative infection alone, 23.9% had Gram-positive infection alone, and 21.7% had mixed Gram-positive and Gram-negative infections, and these patients were equally distributed within the three groups Table 4. Of all patients, 38.3% were bacteremic (Gram negative 47.2%, Gram positive 33.5%, Gram negative plus Gram positive 5.6%, fungi 4%, others/other combinations 9.7%). The protocol required that patients have evidence of organ/system hypoperfusions or dysfunction at time of randomization, and 60% of patients had two or three organ/systems involved. The three most common abnormalities noted were increase of plasma lactate concentration or evidence of metabolic acidosis (74%), oliguria (59%), and hypoxemia (58%). There were no significant differences in the number or type of organ/system hypoperfusion between the three groups (data not shown). Surgery was performed within 3 days before infusion in 42%, 42%, and 41% of the patients in the high-dose, low-dose, and placebo groups, respectively.

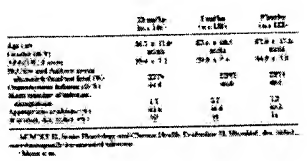


Table 2. Baseline characteristics of patients in shock who received 15 or 3 mg/kg monoclonal antibody to recombinant human tumor necrosis factor-alpha (BAY tim placebo. There were no patients in the McCabe and Jackson rapidly fatal group. patients had no underlying condition

Table 3. Distribution of infection sites for patients in shock who received 15 or 3 r murine monoclonal antibody to recombinant human tumor necrosis factor-alpha (1351) or placebo

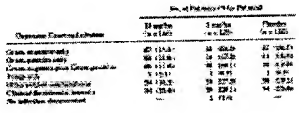


Table 4. Distribution of causative organisms in patients in shock who received 15 of murine monoclonal antibody to recombinant human tumor necrosis factor-alph times 1351) or placebo

Preinfusion plasma concentrations (median [25% to 75% range]) of both TNF-alpha and IL-6 were higher in shock patients (n = 379) than in patients not in shock (n = 125): shock TNF-alpha concentration of 83 (40 to 252) pg/mL, shock IL-6 concentration of 2.86 (0.77 to 23.47) ng/mL; nonshock TNF-alpha concentration of 55 (21 to 124) pg/mL, nonshock IL-6 concentration of 1.2 (0.4 to 4.7) ng/mL. These differences were statistically significant: p = .0005 for the comparison of TNF concentrations, and p = .0001 for the comparison of IL-6 concentrations. One hour after infusion, plasma TNF-alpha concentrations (pg/mL) had decreased in the two treated groups, but not in the placebo group: placebo group 70 (37 to 183) to 71 (41 to 214) (n = 121); 3-mg/kg group 82 (40 to 271) to 17 (7.5 to 70) (n = 119, p = .0001); 15-mg/kg group 119 (45 to 315) to 7.5 (7.5 to 16) (n = 127, p = .0001).

Outcome. TOP

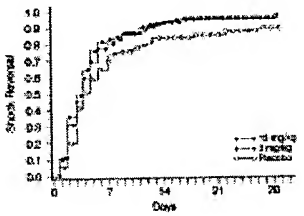
The 28-day, all-cause mortality rate was 42.9% (57/133) in the placebo group. In the low-dose group, the mortality rate was 36.7% (51/139), a reduction of 14.5%, while in the high-dose group, the mortality rate was 44.6% (66/148), an increase of 4%; neither of these differences was statistically significant Table 1. Furthermore, there were no significant differences between the survival rate curves of the three groups (log-rank analysis 3 mg/kg vs. placebo, p = .63 region adjusted, p = .38 unadjusted for region) (Figure 1, bottom).

Examination of the survival rate, based on APACHE II scores at the time of enrollment, showed that scores of <or=to 24 were associated with a greater reduction in the 28-day mortality rate in the 3-mg/kg group than scores of >24: 30 (36%) of 84, 18 (22%) of 82, and 25 (35%) of 72 compared with 36 (56%) of 64, 32 (57%) of 56, and 31 (53%) of 59 for the 15-mg/kg, 3-mg/kg, and placebo groups, respectively. A comprehensive multivariate analysis will be reported separately.

Shock Reversal. TOP

The prospectively defined efficacy analysis stated that we wished to compare ``patients who survive through day 7 and the subset of these patients who survive through day 28" to determine the proportion of patients in whom shock reversal occurred. Only first episodes of shock were included in the analysis. The numbers of patients in whom shock resolved within 7 days after infusion were: 85 (78%) of 109, 81 (78.6%) of 103, and 68 (70.1%) of 97 in the 15-mg/kg, 3-mg/kg, and placebo groups, respectively. The p value for the comparison of either treatment with placebo is 0.15. The same effect is apparent when comparing the subgroup of patients who survived 28 days; life-table analysis showed a clear separation, in particular between the high-dose group and the placebo group. The more rapid reversal of shock was statistically significant for both dose groups (15 mg/kg vs. placebo log rank p = .007; 3 mg/kg vs. placebo p = .01, Figure 2, Table 5.

Figure 2. Kaplan-Meier plot showing time to reversal of shock for patients with shock at the time of randomization and infusion, surviving 28 days. Table 5 shows the number of patients in each group at risk for the event on



days 0, 7, 14, and 28. The p values, adjusted (and unadjusted) for region (a region is a cluster of centers), were: 15 mg/kg vs. placebo--log rank 0.003 (0.007), Wilcoxon 0.005 (0.01); 3 mg/kg vs. placebo--log rank 0.03 (0.01), Wilcoxon 0.03 (0.04).

	Day			
	0	7	14	28
15 mg/kg	81	10	2	1
3 mg/kg	87	12	3	0
Placebo	75	19	11	7

Table 5. Number of patients in each group (15 or 3 mg/kg of murine monoclonal a recombinant human tumor necrosis factor-alpha [BAY times 1351] or placebo) at shock on days 0, 7, 14, and 28

Organ System Failure. TOP

Regardless of the patients' baseline organ failure status, the number of patients developing at least one organ failure during the 28-day study period was as follows: 15-mg/kg group-75 (50.7%) of 148; 3-mg/kg group-74 (53.2%) of 139; placebo group-83 (62.4%) of 133 (3 mg/kg vs. placebo p = .16, 15 mg/kg vs. placebo p = .07). When only those patients who survived the full 28 days are considered, the following number of patients developed at least one organ failure: 15-mg/kg group-33 (40.2%) of 82; 3-mg/kg group-39 (44.3%) of 88; placebo group-45 (59.2%) of 76 (3 mg/kg vs. placebo p = .06, 15 mg/kg vs. placebo p = .03). Of the six organ/system failures identified prospectively, the category ``pulmonary, nonacute respiratory distress syndrome" occurred most frequently, probably a reflection of the fact that the criteria for this category are relatively easy to satisfy (e.g., tachypnea of >or=to50 breaths/min, or mechanical ventilation for >or=to3 days). Similarly, disseminated intravascular coagulation was the second most common system failure, and this diagnosis too required only rather modest changes in platelet count and coagulation times. Nevertheless, comparison of the three groups showed that the overall frequency of organ system failure was greater in the placebo group for all categories as compared with the 3-mg/kg group, and for all but one category when compared with the 15-mg/kg group Table 6. In addition, life-table analysis allowed comparison of the rate at which the organ failures developed. Figure 3 shows a significant delay to the time of onset of first organ failure in patients surviving 28 days in the treatment groups (log-rank statistic, 15 mg/kg vs. placebo p = .03, 3 mg/kg vs. placebo p = .07) Table 7.

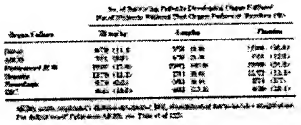


Table 6. Development of organ failure among patients in shock who received 15 of murine monoclonal antibody to recombinant human tumor necrosis factor-alph times 1351) or placebo, and who survived 28 days

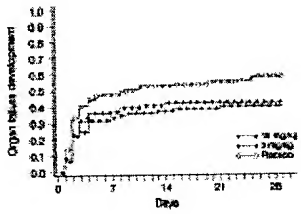


Figure 3. Kaplan-Meier plot showing the time to onset of the first organ/system failure in patients with shock who did not have that organ/system failure at baseline and surviving 28 days. Table 7 shows the number of patients in each group at risk for the event on days 0, 7, 14, and 28. The p values, adjusted (and unadjusted) for region, were: 15 mg/kg vs. placebo--log rank 0.03 (0.03), Wilcoxon 0.03 (0.03); 3 mg/kg vs. placebo--log rank 0.08 (0.07), Wilcoxon 0.06 (0.07).

	Days			
	0	7	14	28
15 mg/kg	82	53	49	45
3 mg/kg	88	52	49	49
Placebo	76	38	35	31

Table 7. Number of patients in each group (15 or 3 mg/kg of murine monoclonal a recombinant human tumor necrosis factor-alpha [BAY times 1351] or placebo) at organ/system failure on days 0, 7, 14, and 28

Another indication of the effect of an intervention in sepsis might be given by the frequency with which organ failures resolved. One measure of this frequency is shown in Table 8 as the proportion of patients in whom an organ failure was present at baseline and subsequently resolved, without recurrence. Here there are only two categories with numbers large enough to merit examination: pulmonary nonacute respiratory distress syndrome and disseminated intravascular coagulation. Of these categories, a slightly greater proportion of patients in the BAY times 1351-treated groups than in the placebo group have resolution of organ failure.

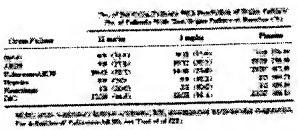


Table 8. Resolution of organ failure among patients in shock who received 15 or 3 murine monoclonal antibody to recombinant human tumor necrosis factor-alpha (1351) or placebo, and who survived 28 days.

Outcome by Infecting Organism. TOP

Overall, there were no differences in mortality rates between the three groups for patients in whom infection had been microbiologically confirmed, and in those patients with only clinically documented infections (data not shown). We were particularly interested to determine whether the type of infection had an effect on outcome. For patients with pure Gram-negative infections, the proportion of patients dying was 11 (40.7%) of 27, 11 (34.4%) of 32, and 11 (31.4%) of 35 in the high-dose, low-dose, and placebo groups, respectively. For patients with pure Gram-positive infections, the figures were 15 (46.9%) of 32, 8 (33.3%) of 24, and 11 (52.4%) of 21, respectively. Similar analyses comparing patients with mixed bacterial infections or nonbacterial infections, or comparing outcome by organism rather than by patient, demonstrated no statistically significant differences between the three groups (data not shown), although the numbers involved were small.

Safety. TOP

For all infused patients, adverse events were reported for 66 (32.2%) of 205 patients treated with 15 mg/kg of BAY times 1351, 54 (29.8%) of 181 patients treated with 3 mg/kg of BAY times 1351, and 48 (28.7%) of 167 patients receiving placebo. Fewer than half of the adverse events were considered by investigators to be possibly or probably drug related, and the differences between the groups were not significant. A serum sickness-like reaction occurred in three patients receiving the high dose of the antibody, and transient hypotension (usually during the infusion of the drug) was reported in 16, nine, and nine patients in the high-dose, low-dose, and placebo groups, respectively. There were no other differences between the active and placebo groups. Approximately 90% of patients receiving monoclonal antibody developed human antimouse antibodies.

Under physiologic conditions, TNF-alpha participates in normal host defenses against infection. Therefore, it was important to determine if the use of the anti-TNF monoclonal antibody had any effect on the clinical or bacteriological response of the baseline infection, or on the development or resolution of secondary infections. The proportion of baseline infections responding clinically and bacteriologically was not different between the three groups of all infused patients, nor were there any differences in the number of patients developing a secondary infection (36.1%, 38.7%, and 39.5% for the high-dose, low-dose, and placebo groups, respectively). Furthermore, for secondary infections that were microbiologically documented, there were no differences between the three groups in the proportions of secondary infections that were bacteriologically eradicated. Preliminary analysis gives no indication that the antibody was associated with an increase in infections caused by intracellular organisms.

DISCUSSION TOP

The principal finding of this study was that in patients with septic shock, administration of an anti-TNF-alpha monoclonal antibody did not reduce the 28-day, all-cause mortality rate. In patients receiving the low dose of anti-TNF antibody (3 mg/kg), there was a 14.5% reduction in the mortality rate, although this decrease was not statistically significant. However, administration of the antibody led to the following results: a) a significant reduction in the time to reversal of shock; b) a significant reduction in the development of organ failure; and c) a significant delay in the onset of first organ failure. These results are of note because they are the first in a study of this kind to show a favorable effect in a group of patients defined prospectively. Furthermore, the findings lend support to the experimental data implicating TNF-alpha as one of the central mediators of septic shock.

Why did INTERSEPT show a beneficial effect on shock reversal and organ failure but no significant reduction in the overall mortality rate? Two possible explanations merit consideration. First, the effects may have been of insufficient magnitude to be translated into a measurable effect on the mortality rate. Second, it can be argued that the study, as it was eventually done, was not of sufficient power. The initial plan called for the enrollment of nearly 1,000

evaluable patients. However, after the decision to study only patients with shock, it was decided that simply extending the trial would have been impracticable. As a result, the shock cohort of 420 patients was too small to demonstrate statistically significant reductions in the mortality rate, although trends in that direction are seen. The effects of the treatment on the secondary end points (shock reversal and organ failure) need to be interpreted with caution. First, although these end points were prospectively defined, it might be argued that we should have used more rigorous tests of statistical significance. Second, the data were censored, i.e., they excluded patients who died before they might have reached the secondary end point. While this situation tends to highlight secondary effects, the inclusion of patients who died may be equally misleading by obscuring potentially important data. There is no consensus in the scientific community as to the most appropriate method of analysis for these types of data, but we believe our interpretation to be valid, although with limitations.

The results from both INTERSEPT and NORASEPT suggest that in patients with shock, there is no additional survival benefit from using the high-dose regimen compared with the low-dose regimen. In NORASEPT, there was an early effect on survival that was not seen in INTERSEPT. However, in other respects, the results of the two studies were generally consistent for the low-dose groups. For example, the 28-day mortality rates for patients in shock in INTERSEPT and NORASEPT, respectively, were: low-dose monoclonal antibody 36.7% and 37.8% vs. placebo 42.9% and 45.6%. The low dose used in INTERSEPT was less than half the low dose used in NORASEPT, raising the question of whether still lower doses might be worth evaluation. In INTERSEPT, both the high- and low-dose groups were more beneficial than placebo in their effects on shock reversal and organ failure, but there was no apparent survival advantage in the high-dose group, despite the greater decrease in TNF concentrations achieved. The reasons for this finding are unclear, but may again be a result of small sample size or reflect the possible harmful consequences in some patients of excessive neutralization of TNF. Finally, if the analysis of INTERSEPT is based only on those patients in shock who were assessed to be in full compliance with the study protocol (the so-called valid cohort, which was 85.5% of all infused shock patients), the reduction in the mortality rate relative to placebo for the low-dose anti-TNF regimen is enhanced, whereas the mortality rate for the high-dose regimen increases: 15-mg/kg group 58 (46.0%) of 126; 3-mg/kg group 40 (33.6%) of 119; placebo group 47 (41.2%) of 114 (log-rank statistic 3 mg/kg vs. placebo p = .46 region adjusted, p = .23 unadjusted for region). For all infused patients, the major reasons for invalidity were: a) onset of sepsis >16 hrs before infusion (n = 22); b) no clear evidence of infection (n = 20); c) absence of temperature abnormality (n = 14); d) no evidence of organ hypoperfusion (n = 7); and e) patient was receiving corticosteroids or pentoxifylline (n = 7).

Although we have focused on patients with shock, we have presented a summary of the results in the entire group because we are aware the entire group data will be of interest. The results in the nonshock group merit a brief comment. Because TNF-alpha is a natural part of host defenses against infection, there was some concern that excessive or prolonged neutralization might be harmful. Table 1 shows that in nonshock patients, there was an increase in the mortality rate in the 15-mg/kg group (36.8% vs. 26.5% in the placebo group). This difference was not statistically significant, although it mirrored the effect seen in the NORASEPT trial for nonshock patients, and was the reason that further enrollment of these patients was halted. Examination of the case record forms from these patients does not show a single cause of death occurring with undue frequency, and it is impossible to be certain if patients receiving the high-dose regimen would have had a significantly higher mortality rate if enrollment had continued. Table 1 also shows what appears to be a very marked reduction in the mortality rate for nonshock patients in the low-dose group of INTERSEPT, almost certainly a consequence of the small numbers in this cell.

Epidemiologic studies [24,25] of patients with shock confirm that Gram-negative and Gram-positive infections occur with almost equal frequency, and cause similar morbidity and mortality. Since there were experimental data showing that TNF-alpha might be a mediator common to both groups of organisms [26], we were particularly interested to see if the underlying infection influenced the efficacy of anti-TNF. No differences were found, a result of some practical importance, since, if the agent were shown to be of benefit, there might be no need for preliminary screening of patients to try and identify, on clinical grounds, subgroups with particular microbiological characteristics.

Despite concerns that neutralizing TNF might impair host responses, there was no increase in secondary infections in the active treatment groups, nor any suggestion of a delayed response to infection. Furthermore, there was no indication that intra-abdominal infections fared worse than infections at other sites, as had been suggested by some preclinical data [27]. Treatment with anti-TNF monoclonal antibody was without significant toxicity, although, not surprisingly, almost all patients developed antimouse antibodies. It remains to be seen if these antibodies cause any difficulties, but, in the future, it may be possible to avoid the problem by using ``humanized" antibodies [28].

During the last 3 yrs, several large clinical trials of adjunctive therapy have been performed in patients with sepsis (reviewed by Lynn and Cohen [3]). These trials have included studies of monoclonal antibodies to endotoxin, pharmacologic inhibitors of platelet-activating factor, and naturally occurring cytokine inhibitors such as IL-1 receptor antagonist. In each case, a body of experimental data implicated the target molecule as a mediator of sepsis. However, when the clinical data were analyzed, efficacy could only be demonstrated in subsets of patients, some of which were identified retrospectively. When these studies were repeated, it was not possible to confirm the initial

findings. The failure of INTERSEPT and other trials of adjunctive agents in sepsis to significantly reduce mortality has raised a number of important questions about our understanding of the pathogenesis of sepsis [29]. Is it due to a fundamental flaw in the approach? Is it naive to imagine that blocking or neutralizing just one component of a complex pathway will be sufficient to substantially alter the mortality rate of a syndrome that occurs, for the most part, in patients with severe and often life-threatening underlying disease? Alternatively, are there problems with the trial design? Were our initial expectations of a therapeutic benefit too optimistic, and therefore, the studies underpowered to detect lower--but perhaps still clinically meaningful--reductions in the mortality rate? Are the criteria for septic shock specific enough, and are we selecting the right patients? Are we using the correct dose, and have we considered the possible need for multiple doses? Some have argued that the 28-day mortality rate alone may not be the only valid end point, and that measures of morbidity may provide additional information. These issues need to be resolved and a consensus needs to be reached if progress is to be made. Meanwhile, INTERSEPT provides important clinical data confirming the experimental evidence implicating TNF-alpha as a central mediator of sepsis. The therapeutic applicability of these data needs to be confirmed by another large trial of monoclonal anti-TNF, and this trial is currently in progress.

ACKNOWLEDGMENTS TOP

We thank all of the patients who participated in this trial, as well as the investigational site study coordinators and nursing staff, without whose dedication and expertise the study could not have been performed.

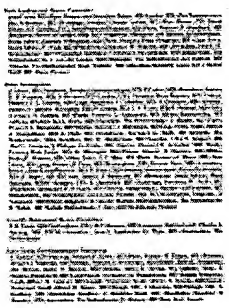


Table 9. International Sepsis Trial (INTERSEPT) Study Group

REFERENCES TOP

1. Bone RC: Gram-negative sepsis: A dilemma of modern medicine. Clin Microbiol Rev 1993; 6:57-68
[Medline Link] [Context Link]

2. Parrillo JE: Pathogenetic mechanisms of septic shock. N Engl J Med 1993; 328:1471-1477
[Context Link]

3. Lynn WA, Cohen J: Adjunctive therapy for septic shock: A review of experimental approaches. Clin Infect Dis 1995; 20:143-158
[Context Link]

4. Waage A, Halstensen A, Espevik T: Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease. Lancet 1987; i:355-357
[Context Link]

5. Girardin E, Grau GE, Dayer J, et al: Tumor necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. N Engl J Med 1988; 319:397-400
[Medline Link] [Context Link]

6. Michie HR, Manogue KR, Spriggs DR, et al: Detection of circulating tumor necrosis factor after endotoxin administration. N Engl J Med 1988; 318:1481-1486
[Medline Link] [Context Link]

7. Tracey KJ, Beutler B, Lowry SF, et al: Shock and tissue injury induced by recombinant human cachectin. Science 1986; 234:470-474
[Medline Link] [Context Link]

8. Tracey KJ, Fong Y, Hesse DG, et al: Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature 1987; 330:662-664
[Medline Link] [CrossRef] [Context Link]

9. Hinshaw LB, Tekamp-Olson P, Chang AC, et al: Survival of primates in LD sub 100 septic shock following therapy with antibody to tumor necrosis factor (TNF alpha). Circ Shock 1990; 30:279-292
[Medline Link] [Context Link]

10. Jesmok G, Lindsey C, Duerr M, et al: Efficacy of monoclonal antibody against human recombinant tumor necrosis factor in E. coli-challenged swine. Am J Pathol 1992; 141:1197-1207
[Medline Link] [Context Link]

11. Fiedler VB, Ingo L, Sander E, et al: Monoclonal antibody to tumor necrosis factor-alpha prevents lethal endotoxin sepsis in adult rhesus monkeys. J Lab Clin Med 1992; 120:574-588
[Medline Link] [Context Link]

12. Silva AT, Bayston KF, Cohen J: Prophylactic and therapeutic effects of a monoclonal antibody to tumor necrosis factor-alpha in experimental Gram negative shock. J Infect Dis 1990; 162:421-427
[Medline Link] [Context Link]

13. Exley AR, Cohen J, Buurman WA, et al: Monoclonal antibody to TNF in severe septic shock. Lancet 1990; 335:1275-1277
[Medline Link] [Context Link]

14. Vincent J, Bakker J, Marecaux G, et al: Administration of anti-TNF antibody improves left ventricular function in septic shock patients. Results of a pilot study. Chest 1992; 101:810-815
[Medline Link] [Context Link]

15. Fisher CJ, Opal SM, Dhainaut JF, et al: Influence of an anti-tumor necrosis factor monoclonal antibody on cytokine levels in patients with sepsis. Crit Care Med 1993; 21:318-327
[Medline Link] [Context Link]

16. Saravolatz LD, Wherry JC, Spooner C, et al: Clinical safety, tolerability, and pharmacokinetics of murine monoclonal antibody to human tumor necrosis factor-alpha. J Infect Dis 1994; 169:214-217
[Context Link]

17. Abraham E, Wunderink R, Silverman H, et al: Efficacy and safety of monoclonal antibody to human tumor necrosis factor-alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial. JAMA 1995; 273:934-941
[CrossRef] [Context Link]

18. Bone RC, Fisher CJ, Clemmer TP, et al: Sepsis syndrome: A valid clinical entity. Crit Care Med 1989; 17:389-393
[Context Link]

19. Emerson TEJ, Lindsey DC, Jesmok G, et al: Efficacy of monoclonal antibody against tumor necrosis factor alpha in an endotoxemic baboon model. Circ Shock 1992; 38:75-84
[Medline Link] [Context Link]

20. Knaus WA, Draper EA, Wagner DP, et al: APACHE II; a severity of disease classification system. Crit Care Med 1985; 13:818-829
[Medline Link] [Context Link]

21. McCabe WR, Jackson GG: Gram negative bacteremia. 1. Etiology and ecology. Arch Intern Med 1962; 110:847-855
[Context Link]

22. Tran DD, Groenveld AB, Meulen JV, et al: Age, chronic disease, sepsis, organ system failure, and mortality in a medical intensive care unit. Crit Care Med 1990; 18:474-479
[Medline Link] [Context Link]

23. Aarden LA, de Groot ER, Schaap OL, et al: Production of hybridoma growth factor by human monocytes. Eur J Immunol 1987; 17:1411-1416
[Medline Link] [Context Link]

24. Ispahani P, Pearson NJ, Greenwood D: An analysis of community and hospital-acquired bacteraemia in a large teaching hospital in the United Kingdom. QJM 1987; 63:427-440
[Context Link]

25. Rayner BL, Willcox PA: Community-acquired bacteraemia; a prospective survey of 239 cases. QJM 1988; 69:907-919
[Context Link]

26. Freudenberg MA, Galanos C: Tumor necrosis factor alpha mediates lethal activity of killed Gram-negative and Gram-positive bacteria in D-galactosamine-treated mice. Infect Immun 1991; 59:2110-2115
[Medline Link] [Context Link]

27. Stack AM, Saladino RA, Thompson C, et al: Failure of prophylactic and therapeutic use of a murine anti-tumor necrosis factor monoclonal antibody in Escherichia coli sepsis in the rabbit. Crit Care Med 1995; 23:1512-1518
[Fulltext Link] [CrossRef] [Context Link]

28. Dhainaut JF, Vincent J, Richard C, et al: CDP571, a humanized antibody to human tumor necrosis factor-alpha: Safety, pharmacokinetics, immune response, and influence of the antibody on cytokine concentrations in patients with septic shock. Crit Care Med 1995; 23:1461-1469
[Fulltext Link] [CrossRef] [Context Link]

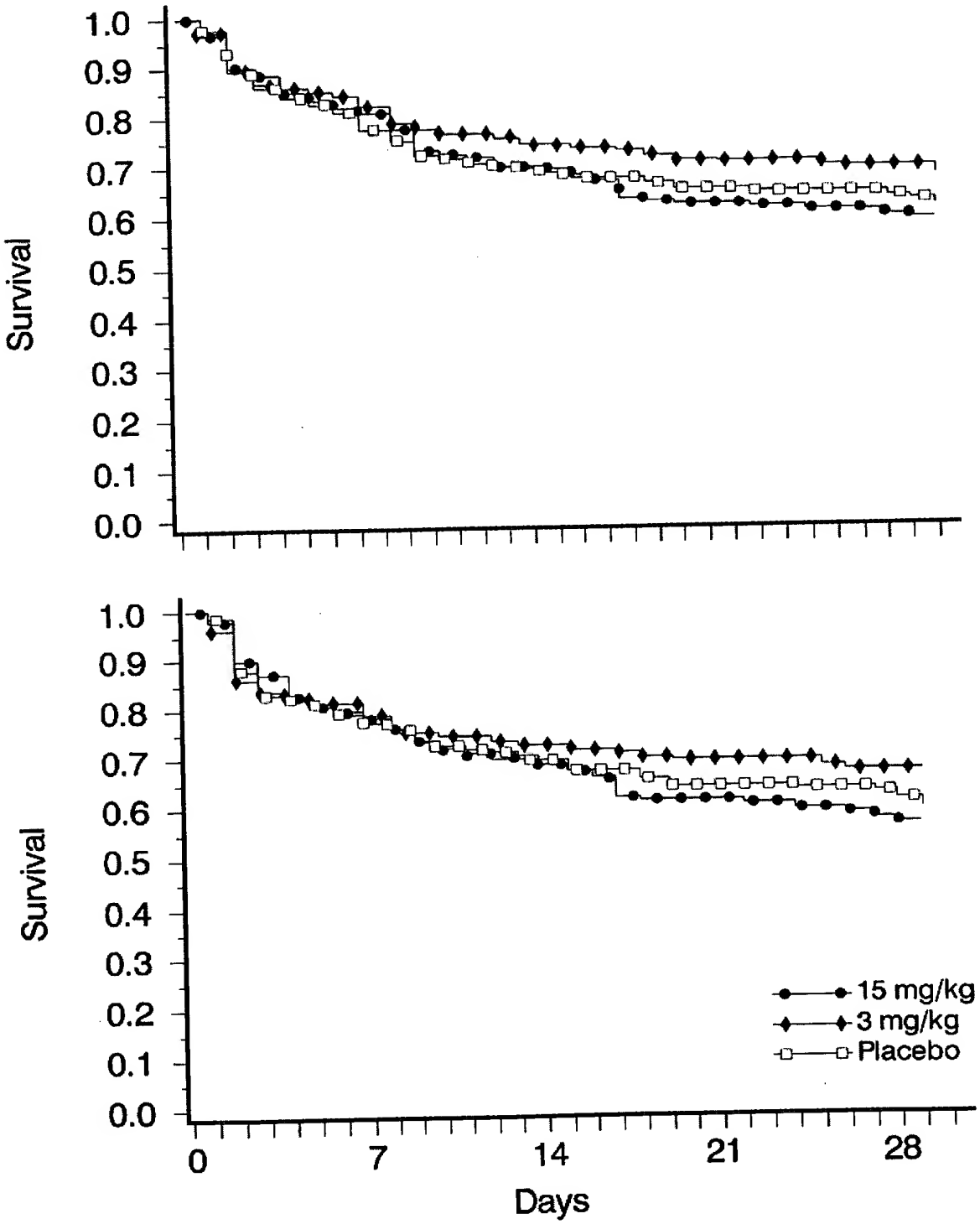
29. Bone RC. Toward a theory regarding the pathogenesis of the systemic inflammatory response syndrome: What we do and do not know about cytokine regulation. Crit Care Med 1996; 24:163-172

Close

Organism Causing Infection	No. of Patien	
	15 mg/kg (n = 148)	3 (n
Gram-negative only	27 (18.2)	3
Gram-positive only	32 (21.6)	2
Gram-negative plus Gram-positive	26 (17.6)	2
Fungi only	5 (3.4)	1
Others/other combinations	24 (16.2)	2
Clinical documentation only	34 (23.0)	2
No infection documented	—	2

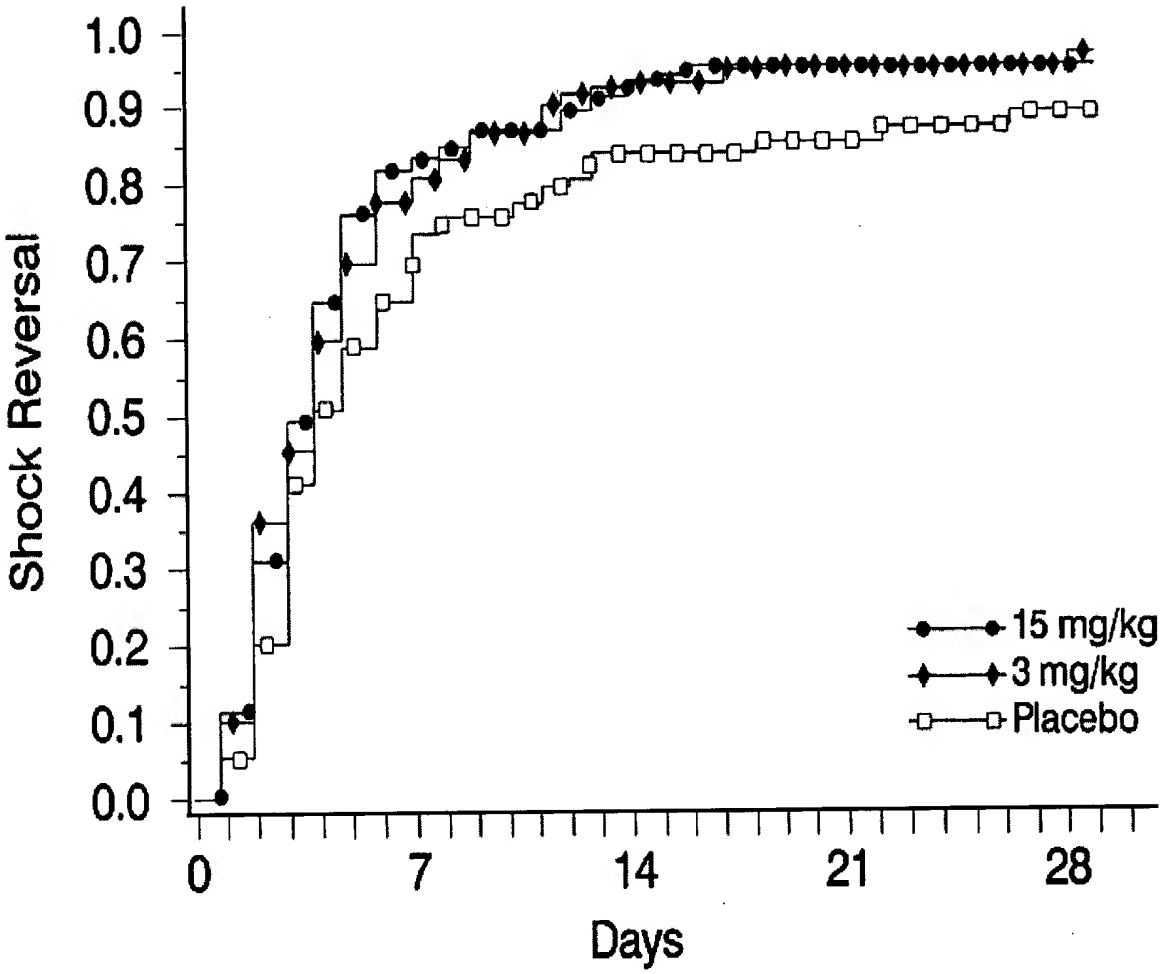
. Distribution of causative organisms in patients in shock who received 15 or 3 mg/kg of murine monoclonal antibody to recombinant human tumor necrosis factor-alpha (BAY times 1351) or placebo

Figure 1



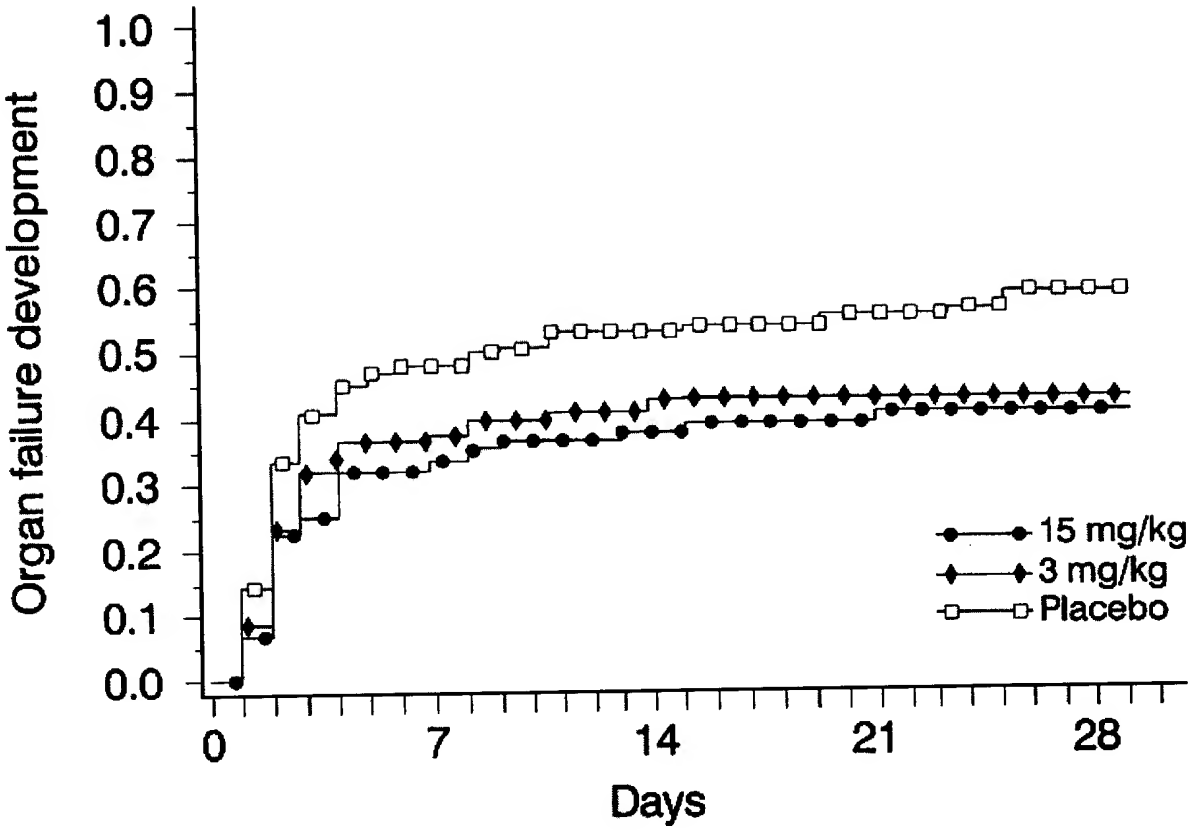
. Top: Kaplan-Meier survival plot for all infused patients. The p values, adjusted (and unadjusted) for region, were: 15 mg/kg vs. placebo--log rank 0.48 (0.52), Wilcoxon 0.54 (0.59); 3 mg/kg vs. placebo--log rank 0.41 (0.19), Wilcoxon 0.39 (0.22). Bottom: Kaplan-Meier survival plot for patients in shock. The p values, adjusted (and unadjusted) for region (a region is a cluster of centers), were: 15 mg/kg vs. placebo--log rank 0.69 (0.77), Wilcoxon 0.69 (0.82); 3 mg/kg vs. placebo--log rank 0.63 (0.38), Wilcoxon 0.62 (0.48).

Figure 2



. Kaplan-Meier plot showing time to reversal of shock for patients with shock at the time of randomization and infusion, surviving 28 days. shows the number of patients in each group at risk for the event on days 0, 7, 14, and 28. The p values, adjusted (and unadjusted) for region (a region is a cluster of centers), were: 15 mg/kg vs. placebo--log rank 0.003 (0.007), Wilcoxon 0.005 (0.01); 3 mg/kg vs. placebo--log rank 0.03 (0.01), Wilcoxon 0.03 (0.04).

Figure 3



. Kaplan-Meier plot showing the time to onset of the first organ/system failure in patients with shock who did not have that organ/system failure at baseline and surviving 28 days. shows the number of patients in each group at risk for the event on days 0, 7, 14, and 28. The p values, adjusted (and unadjusted) for region, were: 15 mg/kg vs. placebo--log rank 0.03 (0.03), Wilcoxon 0.03 (0.03); 3 mg/kg vs. placebo--log rank 0.08 (0.07), Wilcoxon 0.06 (0.07).

Table 2

		Died/Total (%)		
	No.	15 mg/kg	3 mg/kg	Placebo
All Infused	533	87/205 (42.4)	57/181 (31.5)	66/167 (39.5)
Percent change		↑7.3	↓20.3	
χ^2 (CMH) p values vs. placebo		.41 (.45)	.19 (.13)	
Patients in Shock	420	66/148 (44.6)	51/139 (36.7)	57/133 (42.9)
Percent change		↑4.0	↓14.5	
χ^2 (CHM) p values vs. placebo		.67 (.73)	.34 (.30)	
Patients Not in Shock	133	21/57 (36.8)	6/42 (14.3)	9/34 (26.5)
Percent change		↑38.9	↓46.0	
χ^2 (CMH) p values vs. placebo		.29 (.33)	.27 (.17)	

CMH, Cochran-Mantel-Haenszel.

The region-adjusted 95% confidence intervals for the difference in mortality rates for the shock cohort are: 3 mg/kg vs. placebo (–16.7%, 5.7%) and 15 mg/kg vs. placebo (–9.2% , 14.2%). The χ^2 (Cochran-Mantel-Haenszel) p value is shown adjusted for region and (in parentheses) for the unadjusted value.

. Twenty-eight-day, all-cause mortality rates in patients who received 15 or 3 mg/kg of murine monoclonal antibody to recombinant human tumor necrosis factor-alpha (BAY times 1351) or placebo.

Table 2

	15 mg/kg (n = 148)	3 mg/kg (n = 139)	Placebo (n = 133)
Age (yr)	56.7 ± 17.6[a]	57.4 ± 16.8	57.0 ± 17.3
Gender (M/F)	85/63	85/54	80/53
APACHE II score	23.4 ± 7.1	23.0 ± 7.4	24.2 ± 7.3
McCabe and Jackson group ultimately fatal/not fatal (%)	22/74	22/73	23/74
Organ/system failures ≥2 (%)	44.6	44.6	49.6
Mean number of infection sites/patient	1.7	1.7	1.7
Appropriate antibiotic (%)	92.6	94.2	92.5
Microbiol. doc. infect. (%)	77	78	74

APACHE II, Acute Physiology and Chronic Health Evaluation II; Microbiol. doc. infect., microbiologically documented infection.

[a]Mean ± SD.

. Baseline characteristics of patients in shock who received 15 or 3 mg/kg of murine monoclonal antibody to recombinant human tumor necrosis factor-alpha (BAY times 1351) or placebo. There were no patients in the McCabe and Jackson rapidly fatal group. Some patients had no underlying condition

Table 3

Source of Infection (% by Site)	15 mg/kg	3 mg/kg	Placebo
Intra-abdominal	23.5	24.0	22.5
Lower respiratory tract	19.6	23.6	27.7
Bacteremia	22.4	22.7	23.4
Gram-negative only	36.8	49.1	55.6
Gram-positive only	43.9	24.5	33.3
Urinary tract	10.2	9.0	6.1
Skin and soft tissue	5.9	5.6	4.3
Central nervous system	3.1	3.0	1.7
Other	15.3	12.0	14.3

. Distribution of infection sites for patients in shock who received 15 or 3 mg/kg of murine monoclonal antibody to recombinant human tumor necrosis factor-alpha (BAY times 1351) or placebo

Table 4

Organism Causing Infection	No. of Patients (% by Patient) 15 mg/kg (n = 148)	3 mg/kg (n = 139)	Placebo (n = 133)
Gram-negative only	27 (18.2)	32 (23.0)	35 (26.3)
Gram-positive only	32 (21.6)	24 (17.3)	21 (15.8)
Gram-negative plus Gram-positive	26 (17.6)	28 (20.1)	16 (12.0)
Fungi only	5 (3.4)	1 (0.7)	1 (0.8)
Others/other combinations	24 (16.2)	24 (17.3)	26 (19.5)
Clinical documentation only	34 (23.0)	28 (20.1)	34 (25.6)
No infection documented	—	2 (1.4)	—

. Distribution of causative organisms in patients in shock who received 15 or 3 mg/kg of murine monoclonal antibody to recombinant human tumor necrosis factor-alpha (BAY times 1351) or placebo

Table 5

	Day			
	0	7	14	28
15 mg/kg	81	10	2	1
3 mg/kg	87	12	3	0
Placebo	75	18	11	7

. Number of patients in each group (15 or 3 mg/kg of murine monoclonal antibody to recombinant human tumor necrosis factor-alpha [BAY times 1351] or placebo) at risk for shock on days 0, 7, 14, and 28

Table 6

Organ Failure	No. of Surviving Patients Developing Organ Failure/ No. of Patients Without That Organ Failure at Baseline (%)		
	15 mg/kg	3 mg/kg	Placebo
Renal	8/72 (11.1)	7/75 (9.3)	17/66 (25.8)
ARDS	7/71 (9.9)	4/75 (5.3)	7/57 (12.3)
Pulm-non-ARDS	10/37 (27.0)	20/42 (47.6)	20/39 (51.3)
Hepatic	12/79 (15.2)	7/81 (8.6)	11/73 (15.1)
Neurologic	0/78 (0.0)	0/82 (0.0)	2/74 (2.7)
DIC	8/41 (19.5)	9/52 (17.3)	9/39 (23.1)

ARDS, acute respiratory distress syndrome; DIC, disseminated intravascular coagulation. For definition of Pulm-non-ARDS, see Tran et al (22).

. Development of organ failure among patients in shock who received 15 or 3 mg/kg of murine monoclonal antibody to recombinant human tumor necrosis factor-alpha (BAY times 1351) or placebo, and who survived 28 days

Table 7

	Days			
	0	7	14	28
15 mg/kg	82	53	49	48
3 mg/kg	88	52	49	49
Placebo	76	38	35	31

. Number of patients in each group (15 or 3 mg/kg of murine monoclonal antibody to recombinant human tumor necrosis factor-alpha [BAY times 1351] or placebo) at risk for organ/system failure on days 0, 7, 14, and 28

Table 8

Organ Failure	No. of Surviving Patients With Resolution of Organ Failure/ No. of Patients With That Organ Failure at Baseline (%)		
	15 mg/kg	3 mg/kg	Placebo
Renal	6/8 (75.0)	9/12 (75.0)	7/10 (70.0)
ARDS	7/9 (77.8)	10/12 (83.3)	15/19 (78.9)
Pulm-non-ARDS	36/43 (83.7)	34/45 (75.6)	25/37 (67.6)
Hepatic	0/0 (0.0)	5/6 (83.3)	2/3 (66.7)
Neurologic	1/2 (50.0)	3/5 (60.0)	1/2 (50.0)
DIC	37/39 (94.9)	32/35 (91.4)	32/37 (86.5)

ARDS, acute respiratory distress syndrome; DIC, disseminated intravascular coagulation. For definition of Pulm-non-ARDS, see Tran et al (22).

. Resolution of organ failure among patients in shock who received 15 or 3 mg/kg of murine monoclonal antibody to recombinant human tumor necrosis factor-alpha (BAY times 1351) or placebo, and who survived 28 days.

C11

/RESEARCH

Guiding the Selection of Human Antibodies from Phage Display Repertoires to a Single Epitope of an Antigen

Laurent S. Jespers[1], Andy Roberts[2], Stephen M. Mahler[2], Greg Winter[1] and Hennie R. Hoogenboom[1,2,*]

[1]MRC Centre for Protein Engineering, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K.; [2]Cambridge Antibody Technology Ltd., The Science Park, Melbourn, Cambridgeshire SG8 6EJ, U.K. Present address. (L.S.J.) Corvas International N.V., Jozef Plateaustraat 22, B-9000 Gent, Belgium; (S.M.M.) University of New South Wales, P.O. Box 1, Kensington, NSW, Australia. *Corresponding author.

We have developed a strategy for guiding the selection of human antibody fragments from phage display repertoires to a single epitope of an antigen, using rodent monoclonal antibodies as a template. Thus the heavy chain of a rodent antibody (MAb32) directed against human tumor necrosis factor α (TNFα) was cloned and paired as a template chain with a repertoire of human light chains for display as Fab fragments on filamentous phage. The phage were selected by binding to the antigen. The selected human light chains were in turn paired with a repertoire of human heavy chains displayed on phage, and the phage selected again. The isolated phage displaying human antibody fragments binding to TNFα also bound to a peptide comprising the N-terminal region of TNFα as with MAb32. One of the human Fab fragments was recloned for expression as a glycosylated human antibody in mammalian cells: Binding to TNFα could be competed with MAb32 or with anti-serum to the peptide, indicating the same epitope. The human antibody was found to have a binding affinity (K_d=15 nM) similar to MAb32 (K_d=26 nM). The process contrasts with existing means of "humanizing" rodent monoclonal antibodies in that the antibodies derived are completely human.

Received 6 May 1994; accepted 7 June 1994.

It has become possible to make antibodies from large combinatorial repertoires of antibody fragments displayed on filamentous bacteriophage by selection with antigen[1] (for review see ref. 2). Antibodies have been isolated that bind as strongly as those isolated using hybridoma technology[3-7], and against a range of antigens, including haptens[7,8] and foreign antigens[5], self-antigens[6,9], viral antigens[10,11], cell surface antigens[6,12] and even proteins of the lumen of the endoplasmic reticulum such as BiP[13].

The antigenic epitopes recognized by the selected antibodies can be mapped, and this has led to the identification of new epitopes of p53 (ref. 13). However, antibodies are also required against known epitopes, for example against the neutralizing epitope of a virus, and it is therefore desirable to be able to focus the selection of antibodies to a single epitope. Recently antibody heavy and light chains of a mouse monoclonal antibody (MAb) against a hapten were used to isolate human antibodies in which key hapten-binding contacts were retained[14]. Here we have used the same principle, guided selection (or epitope imprinted selection[2]), to make a human antibody against a single epitope of TNFα recognized by the rodent antibody MAb32. The process takes as a starting point one of the large number of rodent MAbs that have already been isolated to many desirable epitopes.

As depicted in Figure 1, the rodent heavy chain is cloned for display as a repertoire of hybrid Fab fragments by combination with a repertoire of human light chains fused to phage. Each phage thus displays the same (mouse) heavy chain in combination with one human light chain from the repertoire. The phage repertoire is then selected with antigen. The human light chains emerging from the selection are then combined with a repertoire of human heavy chains fused to phage, and the phage selected to yield entirely human Fab fragments. The rodent antibody thereby provides a template chain to guide the selection. Alternatively the rodent light chain could also be used as a template.

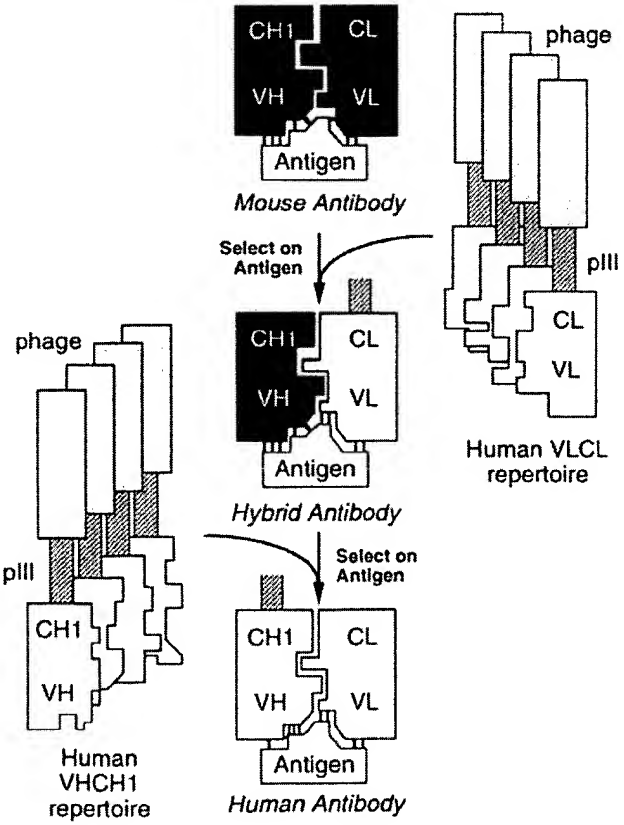


FIGURE 1. The principle of guided selection (see text).

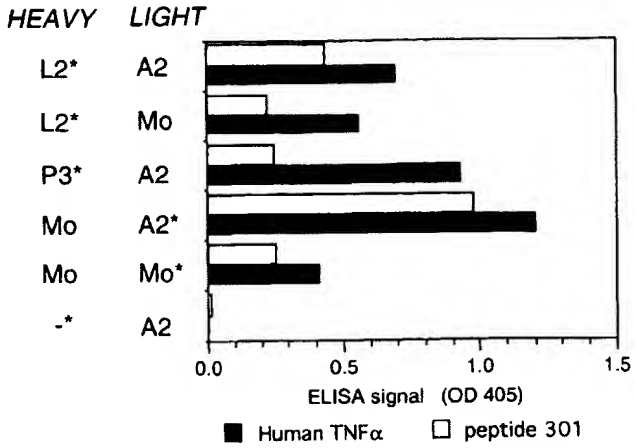


FIGURE 2. Binding of phage displaying Fab fragments to human TNFα and peptide 301 by ELISA. One chain is fused to g3p (indicated by *). Mo (mouse MAb32 heavy or κ light chain), A2 (human λ light chain); P3, L2 (human heavy chains); –, phage fd.

Results

We used a two replicon system[15] for the phage display of Fab fragments. First we displayed Fab fragments from the MAb32 hybridoma. The heavy chain gene (mouse V_H-human $C\mu1$) was cloned into a plasmid vector for secretion of the heavy chain from the bacteria; the light chain gene (mouse $V\kappa$-human $C\kappa$) was cloned into a phage vector for fusion with the phage coat protein (g3p). When the bacteria were infected with the fd-phage, phages were produced that bound to human TNFα. Similar results were obtained when the heavy chain was anchored on phage, and the light chain provided as a soluble fragment. Each chain was then used to guide the selection of human antibodies.

Guided selection. A repertoire of 10^7 human λ light chains fused to phage was used to infect bacteria harboring the soluble MAb32 heavy chain, and the progeny phage selected by binding to TNFα-coated tubes. The selected phage bearing light chains were then used to infect bacteria harboring the soluble mouse heavy chain, and the phage clones were tested for binding to TNFα by ELISA. After the first round of selection, no binders (0/16) were detected; after four rounds most (24/28) phages bound. Three light chains were identified by sequencing the 24 clones, and the sequences, A2, D1 and C4 (Table 1), were found to be derived from the same λ germline segment DPL3 (nomenclature as in ref. 16). We failed to isolate strong binders from a repertoire of 1.4×10^7 human κ chains.

The selected human λ light chains were recloned into a plasmid for expression, and were now used to guide the selection of a repertoire of 5×10^6 human heavy chains fused to phage. Binders were detected after 5 rounds of selection, and sequencing revealed three unique human heavy chains, P1, P2 and P3 (Table 1), derived from the same germline segment DP51 (ref. 17). All three selected V_H-genes had relatively short CDR3 loops (8, 9 and 10 residues), but shared little homology in this sequence.

To show that the mouse light chain was also able to guide the selection, it was paired with the repertoire of human heavy chains fused to phage. After four rounds of selection, two human heavy chains, L2 and L8, derived from the same germline segment DP46 (nomenclature as in ref. 17), were isolated from phage binding to TNFα. The L2 and L8 heavy chains could also be paired with the human A2 light chain to form a TNFα-binding Fab fragment.

Binding specificity and affinity. The phage displaying mouse MAb32 Fab fragment (Mo-Mo* of Fig. 2) bound by ELISA to a peptide (peptide 301), comprising in part the epitope on TNFα (the first 18 residues of human TNFα[18]). All the selected hybrid or human Fab fragments displayed on phage also bound to the peptide, illustrated for several heavy and light chain combinations in Figure 2, indicating that the selection had been guided to a single epitope of TNFα.

The human antibody light (A2) and heavy (P3) chain genes were recloned for production as whole antibody by myeloma cells, and characterized further. Antibody P3A2 (and MAb32) bound to TNFα by ELISA, and no significant binding was detected to keyhole limpet haemocyanin, ovalbumin, cytochrome c, bovine serum albumin, human thyroglobulin, or 2-phenyloxazol-5-one-BSA or to plastic (not shown). The antibody P3A2 appeared to bind to the same epitope of TNFα as MAb32, as its binding could be competed with MAb32 or with sheep anti-301 serum (Fig. 3A and 3B). Furthermore, the P3A2 antibody bound to peptide 301, and (like MAb32) preferentially to TNFα with four N-terminal residues deleted (Fig. 3D).

TABLE 1. Deduced protein sequences of V_H and V_L genes of antibody fragments binding to human TNFα*.

A. Light chains

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
MAb32	AIELTQPAILSASPGGKVTMTC	RASSSVSYMH	WYQQQKPGSSPKPWIY	ATSNLAS	GVPTRFSGSGTGTSYSLTISRVEAEDAATYYC	QQWSRNPFT	FGSGTKLEIK
DPL3	QSVLTQPPSASGTPGQRVTISC	SGSSSNIGSNYVY	WYQQLPGTAPKLLIY	RNNQRPS	GVPDRFSGSKSGTSASLAISGLRSEDEADYYC	AAWDDSLSG	
A2	-------S-V-AA---K-----	--------N----	----------	-------	----------S----------	---------RRVV	--G----TVLG
C4	-------S----------	----------	----------	-------	----------	---------RDVV	--G----TVLG
D1	-------A----------	----------	------R----	-------	----------	---------RDYV	--T---VTVLG

B. Heavy chains

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
MAb32	QVKLQQSGAELVKPGASVKMSCKASGYTFA	SYWIN	WVKQRPGQGLEWIG	HIYPVRSITKYNEKFKS	KATLTLDTSSSTAYMQLSSLTSEDSAVYYCSR	GDSDYYAMDY	WGQGTTVTVSS
DP51	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYSMN	WVRQAPGKGLEWVS	YISSSSSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRDEDTAVYYCAR		
P1	Q---LQ----------	--A-S	----------	----------	----------T----------	SLVGALDY	----L-----
P2	Q----------	-----	----------	------G----------	----------S	SSWYGGYD	----L-----
P3	-----Q----------	-----	----------	------G----------	---------- --------A----------	SVDYGMDV	----------
DP46	QVQLVESGGGVVQPGRSLRLSCAASGFTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
L2	----Q------L-K--G----------	-----	----------	----------	----------K	GGLGTYYYDSSGHKGFDP	----L----L
L8	----------	-----	----------	----------	----------S	GRYCSGGSCSPFDY	----L-----

*mouse MAb32 V-genes and selected human V-genes aligned to their closed germline; identical residues in the selected genes are represented by hyphens (except in CDR3); FR, framework region; CDR, complementary determining region.

The MAb32 antibody is reported to have an affinity of about 9 nM by RIA[19]. Here we determined the affinities by equilibrium capture[20,21] of MAb32 and P3A2 antibodies as 21 nM and 11 nM respectively (Scatchard plot; not shown) or 26 nM and 15 nM (Klotz plot; Fig. 4).

Discussion

We have used the heavy chain of a rodent MAb directed against human TNFα to guide the selection of a repertoire of human Fab fragments displayed on phage. We thereby succeeded in making human Fab fragments directed against human TNFα. The selected phage bound to a peptide (peptide 301) comprising the N-terminal portion of TNFα, suggesting that the selection had been guided to the same epitope recognized by the rodent MAb (Fig. 2). One of the selected human Fab fragments, P3A2, was expressed in mammalian cells as a complete antibody, and also bound to TNFα and peptide 301; the binding was competed with MAb32 or with anti-301 serum. Like MAb32, the binding of antibody P3A2 was enhanced to a mutant of TNFα with an N-terminal deletion of four residues (Fig. 3). It is clear that antibodies MAb32 and P3A2 recognize the same epitope with similar affinities.

We envisage that the complementarity between the surface of the antigen and the mouse template chain helps drive the new contacts made by the repertoire of human chains to the same epitope. It would therefore be expected that the antibody P3A2 has the same "footprint" on the antigen as MAb32 and that the relative orientations of heavy and light are similar. However the molecular contacts from the antibody to the antigen are likely to be different. The L1 loops and L3 loops in P3A2 (λ chain) are longer than the loops from MAb32 (κ chain), and the H3 loop is shorter, indicating that the conformations of three of the antigen binding loops are different. In general, the differences in the structure of the binding site could give rise to differences in recognition of related antigens and of unrelated antigens, and may provide, for example, a means of losing an unwanted cross-reactivity.

The apparent lack of structural homology between MAb32 and the selected human antibodies contrasts with the guided selection of human antibodies to the hapten phenyloxazolone (phOx), where several of the key contacts to hapten were retained[14]. This may reflect the lack of human light chains with similar hypervariable loop conformations to the MAb32 light chain: there are no human Vκ segments with 10 residue L1 loops, as in MAb32 (ref. 22). It may also reflect the differences between binding sites for haptens and protein antigens. Thus haptens are often located in a cavity at the base of the hypervariable loops with contacts to relatively conserved residues, whereas with protein antigens, the contacts are often made at the tips of the hypervariable loops (A. Murzin and C. Chothia, personal communication). Although the presence of structural homology does not appear to be essential, it would presumably be desirable.

It is interesting to compare guided selection with other possible strategies for making rodent or human antibodies against a single epitope using phage technology. In principle, rodent MAbs could be used to screen phages raised against many epitopes by binding competition, or used in the selection process to elute the phage from the antigen. The strategy of guided selection would be expected to impose further constraints, for example the same footprint and orientation of antibody heavy and light chains on the epitope. However, the sequential pairing of each repertoire with a template chain is also likely to sample pairings of human chains rarely encountered even in large repertoires[4,23]. For example, it would be difficult to sample all possible combinations of 10^6 light chains and 10^8 heavy chains of a

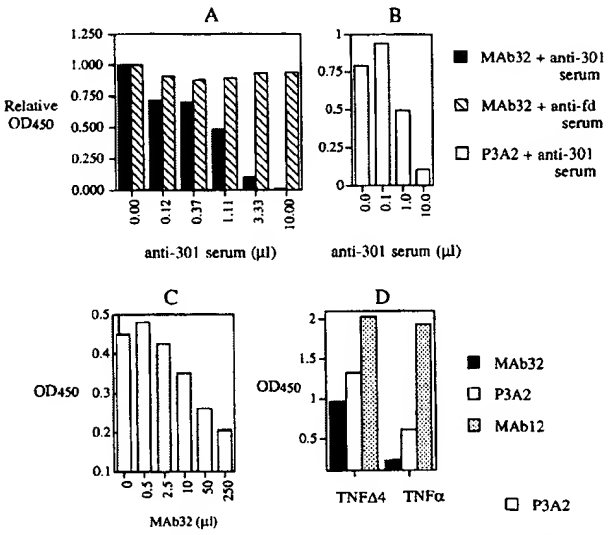


FIGURE 3. Binding of complete antibodies to human TNFα by ELISA. (A) Competition of MAb32 with anti-301 serum or with control antiserum (anti-fd, ref. 1); (B) Competition of P3A2 with anti-301 serum; (C) Competition between P3A2 and MAb32; (D) Binding to TNFα or TNFα with N-terminal deletion of 4 residues (TNFΔ4); MAb12, mouse anti-TNFα antibody binding equally to both forms[19].

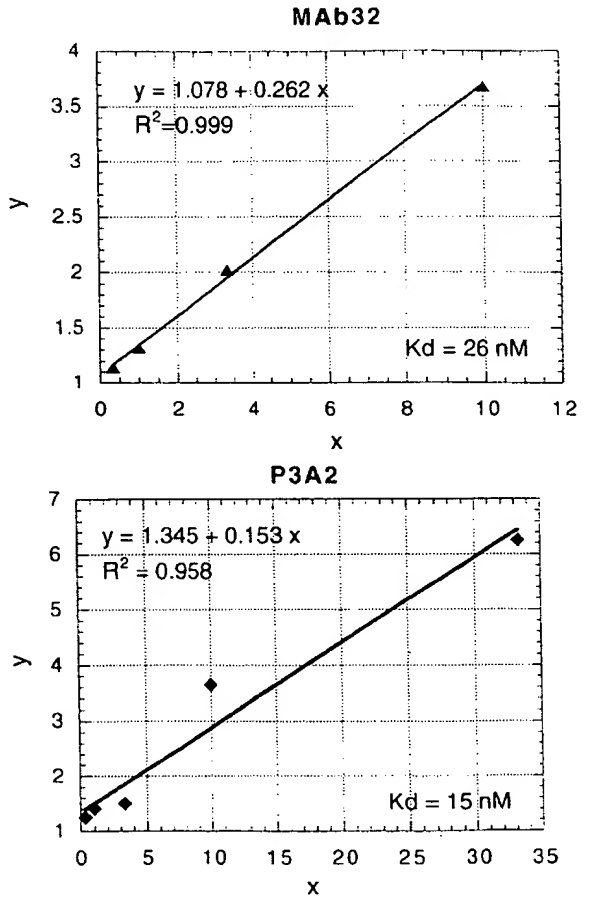


FIGURE 4. Binding of complete antibodies to human TNFα by Keq assay. Plotted is the total antibody concentration divided by the concentration of the complex (y) versus the reciprocal of the total TNFα concentration (nM) (x).

random combinatorial repertoire[24].

Guided selection may also provide an alternative means of humanizing rodent MAbs (for review see ref. 25). Thus in CDR-grafted or reshaped antibodies, the hypervariable loops of a rodent antibody are transplanted to a human antibody[26], so as to recreate the antigen binding site. The epitope and specificity is thus retained, but the antibody includes some rodent sequences. By contrast in guided selection, the antigen binding site and specificity are most likely to differ, the epitope is retained, but the antibodies are entirely human. It remains to be seen whether guided selection wil prove as general as CDR-grafting, but we expect operational rules to emerge with further examples.

Experimental Protocol

Cloning of the V-genes of MAb32. The genes of the mouse MAb32 antibody (α2b, κ; gift from D. Rathjen) were amplified by the polymerase chain reaction (PCR) essentially as described[3]. The mouse V_H and Vκ genes were obtained from the MAb32 cell line, assembled as scFv genes[3], and cloned into phagemid pHEN1 (ref. 15). 9/96 clones secreted soluble scFv fragments binding to TNFα in ELISA, and were sequenced (Table 1). The mouse Vκ and V_H genes were linked to the human Cκ gene or human μ-C_H1 gene respectively by splice overlap extension. The mouse Vκ gene was amplified from scFv-MAb32 DNA with primers MVKBASFI/MJK1FORNX (see below for a complete listing of the sequences of all the oligonucleotides used); the human Cκ gene was obtained by PCR from the mouse-human chimaeric NQ10.12.5 light chain gene[15], with primers MVK-HCK-BACK/HCK-NOT-NM. The two PCR fragments were mixed and amplified with MVKBASFI/HCK-NOT-NM followed by cloning as a SfiI-NotI fragment into pUC19SNmyc (for expression as free light chain[14]), or amplified with MVKBAAPA/HCK-CYSNOT followed by cloning into fd-tet-DOG1[13] as an ApaLI-NotI fragment (for phage display). Similarly, the mouse V_H gene was amplified from scFv-MAb32 with LMB3/MVH1FOR-2 and combined with the human μ-C_H1 gene (obtained from human IgM-derived cDNA[7] with MVH-HCH1/HCM1FONO). The assembled V_H-C_H1 DNA was amplified by appropriately tagged oligonucleotides and cloned into pUC19SNmyc or fd-tet-DOG1 (as above).

Construction of human libraries. κ, λ light chain and μ-specific heavy chain cDNA was made from the mRNA prepared from the peripheral blood lymphocytes containing approximately 10^8 B-lymphocytes from two healthy human donors essentially as in reference 7. The first-strand cDNA synthesis was performed with primers HCM1FOR, HCLFOR and HCKFOR for μ-specific, λ and κ libraries respectively, and the V-genes amplified as decribed[7] with appropriate family-based back and forward primers. Approximately 50 ng of the primary PCR product was reamplified in a second PCR using appropriate oligonucleotides appended with ApaLI and NotI restriction sites for cloning. After PCR, the three repertoires (μ, λ and κ) were digested with ApaLI and NotI and cloned into fd-tet-DOG1, to obtain libraries of 5×10^6 clones for μ-derived V_HC_H1, 1.0×10^7 clones for VλCλ, and 1.4×10^7 clones for VκCκ. The frequency of inserts in each of libraries was >95% as judged by PCR screening of clones using fd-SEQ1 and fd-PCR-BACK[3]. Phages were prepared by growing at 37°C in 2xTY with 15μg/ml tetracyclin, and collected after two rounds of PEG-NaCl precipitation as in ref. 1.

Recloning selected light chains for free expression. Human light chains VλA2, VλD1 and VλC4 were amplified from fd with HVL1BACKSFI and HCL1FORAMBNOT and cloned into pUC19SNmyc[14] (as SfiI-NotI fragments) for expression as free light chains.

Recloning of human V-genes for expression as a complete human antibody. The selected human heavy P3 and light A2 genes were recloned in pSV-derived plasmids pALYS30 and pALYS17 repectively, essentially as described[28,29]. The human VH gene was linked to the human α-1 gene and the human Vλ and Cλ were cloned together (details of cloning on request). After co-transfection of SP2/0 cells with both plasmids, a cell line producing a complete human antibody (α1, λ) was isolated. The cell line was adjusted to protein-free medium, and antibody purified from the supernatant using affinity chromatography on Protein A-Sepharose.

Preparation of phage displaying Fab fragments on the surface. *E. coli* cells containing plasmid encoding antibody heavy or light chain for expression as free chains were infected with a 20-fold excess of phage displaying the partner chain(s) (or, after panning, with 1 ml eluate with variable titre). Phage was prepared from an overnight culture by two rounds of PEG-NaCl precipitation (see refs. 1 and 7 for experimental details).

Selection of TNFα binders. Immuno tubes (Nunc) were coated overnight with 2 ml TNFα (10 μg/ml) in 50 mM bicarbonate buffer (pH 9.6). Recombinant human TNF-α (produced in yeast, specific activity of 3.2×10^7 units/mg) was a gift from D. Rathjen, Peptide Technology (Australia). Incubation with phage, washing and elution conditions were as described in reference 7. *E. coli* expressing the free chain were infected with the eluted phage and grown with tetracyclin (15μg/ml) for amplification of the selected phage population (as described above).

ELISA. Individual phage clones displaying heavy and light chains were assayed for binding by ELISA, by infecting *E. coli* expressing the complementary chain. Phage displaying Fab fragments were prepared from overnight cultures by PEG-NaCl precipitation and 50 μl assayed in ELISA. Human TNF-α (10 μg/ml in 50 mM $NaHCO_3$ buffer, pH 9.6, or PBS) was coated by overnight incubation at 4°C onto plastic ELISA plates (Falcon 3912) at 50 μl/well. After washing with PBS, blocking for 2 hours with 2% dried skimmed milk powder (Marvel), 50 μl phage per well was added to 50 μl 4% Marvel, after which the ELISA was continued (with anti-fd phage serum) as described[7]. For the detection of binding to the MAb32 epitope, peptide 301 (H-VRSSSRTPSDKPVAHVVA-OH; gift from D. Rathjen) was coated by overnight incubation at room temperature (10 μg/ml peptide in PBS; 100 μl/well). The wells were blocked for 2 hours with 3% BSA, and the ELISA continued as above (using incubations with Marvel). For competition and specificity ELISAs with the complete antibodies, human antibody P3A2 purified on Protein A Sepharose (0.2 mg/ml), hybridoma supernatant containing MAb32 (at approximately 1 μg/ml) and anti-301 peptide serum raised in sheep (gift from D. Rathjen) were used. Detection of bound antibody was with either peroxidase-labeled goat anti-mouse (or anti-human) IgG (Fc-specific) reagents.

DNA sequencing. The nucleic acid sequences of selected V-regions were determined by the dideoxy chain termination method[30] using a Sequenase kit (USB) and appropriate sequencing primers. Murine MAb32 V-genes cloned into pHEN1 were sequenced with pHEN-SEQ and LINKSEQ[30]; human V_H genes were sequenced with a primer situated in the human $C_H1(\mu)$ region (HCH1-FORSEQ2), while for human Vλ genes, HLAMBDASEQ was taken. Sequences were analyzed using the program MacVector 4.1 (IBI Kodak, New Haven, CT) and using a database of human V_H and V_λ genes[16,17].

Affinity determinations. The affinities were determined by ELISA to measure an affinity constant as described in references 20 and 21. Measurements were done in triplicate.

Alphabetical list of oligonucleotides:

fd-PCR-BACK	5′-GCG ATG GTT GTT GTC ATT GTC GGC-3′
fd-SEQ1	5′-GAA TTT TCT GTA TGA GG-3′
HCH1-FORSEQ2	5′AGG AAG TCC TGT GCG AGG CAG-3
HCK-NOT-NM	5′-GAG TCA TTC TCG ACT TGC GGC CGC TTA TTA ACA CTC TCC CCT GTT GAA GCT CTT-3′
HCKCYSNOT	5′-GAG TCA TTC TCG ACT TGC GGC CGC ACA CTC TCC CCT GTT GAA GCT CTT-3′
HCKFOR	5′-ACA CTC TCC CCT GTT GAA GCT CTT-3′
HCL1FORAMBNOT	5′-CCA CGA TTC TGC GGC CGC CTA TGA ACA TTC TGT AGG GGT CAC TGT-3′
HCLFOR	5′-TGA ACA TTC TGT AGG GGC CAC TGT CTT-3′
HCM1FONO	5′-CCA CGA TTC TGC GGC CGC CAC TGG AAG AGG CAC GTT CTT TTC TTT-3′
HCM1FOR	5′-TGG AAG AGG CAC GTT CTT TTC TTT-3′
HLAMBDASEQ	5′-GTG TGG CCT TGT TGG CTT G-3′
HUVHBACKAPA	HuVHBACK primers appended with 5′-CAT GAC CAC AGT GCA... (with C as first nucleotide for all six primers) (ref. 7)
HUVκ/λBACKAPA	HuVκ/λBACK primers appended with 5′-TGA GCA CAC AGT GCA CTC... (ref.7)
HVL1BACKSFI	5′-GTC CTC GCA ACT CGC GCC CAG CCG GCC ATG GCC CAG TCT GTG TTG ACG CAG CCG CC-3′
LMB3	5′-CAG GAA ACA GCT ATG AC-3′
MJK1FORNX	5′-CCG TTT GAT TTC CAG CTT GGT GCC-3′
MVH-HCH1	5′-GGG ACC ACG GTC ACC GTC TCC TCA GGA AGT GCA TCC GCC CCA ACC CTT TTC-3′
MVH1FOR-2	5′-TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC-3′
MVHBACKSFI	5′-CAT GCC ATG ACT CGC GGC CCA GCC GGC CAT GGC C(C/G)A GGT (C/G)(A/C)A (A/G)CT GCA G(C/G)A GTC (A/T)GG-3′
MVH1BACKAPA	5′-CAT GAC CAC AGT GCA CAG GTS MAR CTG CAG SAG TCW GG-3′
MVK-HCK-BACK	5′-GGC ACC AAG CTG GAA ATC AAA CGG ACT GTG GCT GCA CCA TCT GTC TTC-3′
MVKBAAPA	5′-CAC AGT GCA CTC GAC ATT GAG CTC ACC CAG TCT CCA-3′
MVKBASFI	5′-CAT GAC CAC GCG GCC CAG CCG GCC ATG GCC GAC ATT GAG CTC ACC CAG TCT CCA-3′
pHEN-SEQ	5′-CTA TGC GGC CCC ATT CA-3′ (with S=C/G, M=A/C, R=A/G and W=A/T)

Acknowledgments

We are grateful for essential materials provided by D. A. Rathjen from Peptide Technology Limited, Australia, and advice in constructing the antibody libraries from J. D. Marks and A. D. Griffiths. L. S. J. was supported by a BRIDGE fellowship from the European Community (S/BIOT-913014); S.M.M. acknowledges support by Peptide Technology Limited.

References

1. McCafferty, J., Griffiths, A. D., Winter, G. and Chiswell, D. J. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature **348**:552–554.
2. Winter, G., Griffiths, A. D., Hawkins, R. E. and Hoogenboom, H. R. 1994. Making antibodies by phage display technology. Ann. Rev. Immunol. **12**:433–455.
3. Clackson, T., Hoogenboom, H. R., Griffiths, A. D. and Winter, G. 1991. Making antibody fragments using phage display libraries. Nature **352**:624–628.
4. Griffiths, A. D., et al. 1994. Isolation of high affinity human antibodies directly from large synthetic repertoires. EMBO J. *In press.*
5. Marks, J. D., Griffiths, A. D., Malmqvist, M., Clackson, T. P., Bye, J. M. and Winter, G. 1992. By-passing immunization: Building high affinity human antibodies by chain shuffling. Bio/Technology **10**:779–783.
6. Barbas, C. F., Languino, L. R. and Smith, J. W. 1993. High-affinity self-reactive human antibodies by design and selection: targeting the integrin ligand binding site. Proc. Natl. Acad. Sci. USA **90**:10003–10007.
7. Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D. and Winter, G. 1991. By-passing immunization: Human antibodies from V-gene libraries displayed on phage. J. Mol. Biol. **222**:581–597.
8. Hoogenboom, H. R. and Winter, G. 1992. By-passing immunisation. Human antibodies from synthetic repertoires of germline VH-gene segments rearranged *in vitro*. J. Mol. Biol **227**:381–388.
9. Griffiths, A. D., Malmqvist, M., Marks, J. D., Bye, J. M., Embleton, M. J., McCafferty, J., Baier, M., Holliger, K. P., Gorick, B. D., Hughes, J. N., Hoogenboom, H. R. and Winter, G. 1993. Human anti-self antibodies with high specificity from phage display libraries. EMBO J. **12**:725–734.
10. Burton, D. R., Barbas, C. F., Persson, M. A. A., Koenig, S., Chanock, R. M. and Lerner, R. A. 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic individuals. Proc. Natl. Acad. Sci. USA **88**:10134–10137.
11. Zebedee, S. L., Barbas, C. F., Hom, Y., Caothien, R. H., Graff, R., Degraw, J., Pyati, J., LaPolla, R., Burton, D. R., Lerner, R. A. and Thornton, G. B. 1992. Human combinatorial antibody libraries to hepatitis B surface antigen. Proc. Natl. Acad. Sci. USA **89**:3175–3179.
12. Marks, J. D., Ouwehand, W. H., Bye, J. M., Finnern, R., Gorick, B. D., Voak, D., Thorpe, S. J., Hughes-Jones, N. C. and Winter, G. 1993. Human antibody fragments specific for human blood group antigens from a phage display library. Bio/Technology **11**:1145–1149.
13. Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D. and Winter, G. 1994. Antibody fragments from a 'single pot' phage display library as immunochemical reagents. EMBO J. **13**:692–698.
14. Figini, M., Marks, J. D., Winter, G. and Griffiths, A. D. 1994. *In vitro* assembly of repertoires of antibody chains by renaturation on the surface of phage. J. Mol. Biol. **239**:68–78.
15. Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P. and Winter, G. 1991. Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. Nucl. Acids Res. **19**:4133–4137.
16. Williams, S. C. and Winter, G. 1993. Cloning and sequencing of human immunoglobulin V lambda gene segments. Eur. J. Immunol. **23**:1456–1461.
17. Tomlinson, I. M., Walter, G., Marks, J. D., Llewelyn, M. B. and Winter, G. 1992. The repertoire of human germline VH sequences reveals about fifty groups of VH segments with different hypervariable loops. J. Mol. Biol. **227**:776–798.
18. Rathjen, D. A. and Aston, R. 1993. Selective enhancement of tumour necrosis factor activity: mapping regions with monoclonal antibodies. Biomed. Chem. Lett. **3**:457–462.
19. Rathjen, D. A., Cowan, K., Furphy, L. J. and Aston, R. 1991. Antigenic structure of human tumour necrosis factor: recognition of distinct regions of TNF alpha by different tumour cell receptors. Mol. Immunol. **28**:79–86.
20. Friguet, B., Chaffotte, A. F., Djavadi, O. L. and Goldberg, M. E. 1985. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. J. Immunol. Methods **77**:305–319.
21. Goldberg, M. E. and Djavadi, O. L. 1993. Methods for measurement of antibody/antigen affinity based on ELISA and RIA. Curr. Opin. Immunol. **5**:278–81.
22. Cox, J. P. L., Tomlinson, I. M. and Winter, G. 1994. A directory of human germ-line V_K segments reveals a strong bias in their usage. Eur. J. Immunol. **24**:827–836.
23. Waterhouse, P., Griffiths, A. D., Johnson, K. S. and Winter, G. 1993. Combinatorial infection and *in vivo* recombination: a strategy for making large phage antibody repertoires. Nucl. Acids Res. **21**:2265–2266.
24. Huse, W. D., Sastry, L., Iverson, S. A., Kang, A.S., Alting, M.M., Burton, D. R., Benkovic, S. J. and Lerner, R.A. 1989. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. Science **246**:1275–1281.
25. Winter, G. and Harris, W. J. 1993. Humanized antibodies. Trends Pharmacol. Sci. **14**:139–143.
26. Jones, P. T., Dear, P. H., Foote, J., Neuberger, M. S. and Winter, G. 1986. Replacing the complementarity-determining regions in a human antibody with those from a mouse. Nature **321**:522–525.
27. Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. and Foeller, C. 1991. Sequences of Proteins of Immunological Interest. 5th Edit. U.S. Department of Health and Human Services, Public Health Service National Institutes of Health.
28. Orlandi, R., Gussow, D. H., Jones, P. T. and Winter, G. 1989. Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. Proc. Natl. Acad. Sci. USA **86**:3833–3837.
29. Foote, J. and Winter, G. 1992. Antibody framework residues affecting the conformation of the hypervariable loops. J. Mol. Biol. **224**:487–499.
30. Sanger, F., Nicklen, S. and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.